THE EFFECTS OF VARIATION IN ELECTRON DONOR CONCENTRATION AND TYPEON DEEP-SEA ENDOSYMBIONT COMMUNITY COMPOSITION AND GENE EXPRESSION

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THE EFFECTS OF VARIATION IN ELECTRON DONOR CONCENTRATION AND TYPEON DEEP-SEA ENDOSYMBIONT COMMUNITY COMPOSITION AND GENE EXPRESSION

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To the students and faculty of the Georgia Institute of Technology's School of Biology

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LIST OF SYMBOLS AND ABBREVIATIONS

γ	Gamma
α	Alpha
HTS	High-Throughput Sequencing
FISH	Fluorescence In-Situ Hybridization
TEM	Transmission Electron Microscopy
H_2S	Hydrogen Sulfide
S_2O_3	Thiosulfate
^{13}C	Carbon 13
OTU	Operational Taxonomic Unit
RDP	Ribosomal Database Project
MEGAN	MEtaGenomeANalyzer
ANOVA	Analysis of Variance

ABSTRACT

Chemosynthetic symbioses are among the most prevalent microbial symbioses found in marine systems. These associations often dominate reducing environments such as hydrothermal vents, where they play critical roles in biogeochemical cycling. Among the diverse number of organisms that participate in chemosynthetic symbioses is *Ifremeria nautilei*, a gastropod found surrounding the deep-sea hydrothermal vents of the South Pacific Ocean. Little is known about how chemosynthetic symbiont community composition and gene expression change in response to gradients of electron donors in the vent environment. Understanding these changes offers significant insight into the environmental conditions and physiological mechanisms necessary to sustain the relationship present between host and symbiont. To address this question, individual *Ifremeria* were collected from the Lau Basin hydrothermal vent system and placed in pressurized, sterile aquaria under the following conditions: i) no electron donor, ii) 100 µM hydrogen sulfide, iii) 300 µM hydrogen sulfide, iv) 300 µM thiosulfate. Stable carbon isotope (^{13}C) incorporation rates were determined for each condition, with 300 µM thiosulfate yielding the highest average rate of carbon incorporation. Amplicon (16S rRNA gene) and metatranscriptomic sequencing were used to compare the phylogenetic diversity and differential gene expression of the symbiotic communities in gill tissue excised from *Ifremeria*in each treatment. Amplicon analyses revealed two major symbiont lineages within the phylum γ-proteobacteria: putative sulfur-oxidizing symbionts of the Chromatiales and methane-oxidizing symbionts of the Methylococcales. Of these, Chromatiales symbionts dominated, consisting of a single operational taxonomic unit (OTU) representing 81.2-99.6% of the symbiont population. Methylococcales symbionts were represented by two distinct OTUs (0.003-17.5% of sequences) and were present in all host individuals, excluding those exposed to 300uM

hydrogen. Preliminary results of the metatranscriptome analysis confirm the expression of genes from both symbiont pools, including genes mediating sulfur oxidation and methane oxidation, despite an assumed lack of methane in the treatments. Genes for sulfur oxidation were ten-fold higher in abundance than those for methane oxidation. These results confirm that *Ifremeria* engages in a "dual" symbiont strategy using thiotrophic and methanotrophic partners and that this community may be sensitive to changes in electron donor availability, due potentially to symbiont competition within the host, host sanctions of symbiont "cheaters," or direct effects of substrate (sulfide) toxicity.

CHAPTER 1

INTRODUCTION

Mutualistic associations between animals and symbiotic bacteria are among the most significant ecological interactions in nature. These associations often result in biological innovation, allowing the partners involved to exploit novel capabilities and function more efficiently together than apart. One of the more prevalent, but still relatively understudied, forms of microbial symbioses is the association between chemoautotrophic bacteria and the invertebrate fauna that inhabit marine environments rich in reduced chemicals. These relationships are more generally known as chemosynthetic symbioses and are based on the exchange of chemosynthetic substrates, notably either reduced sulfur or methane as electron donors, and organic carbon between host and symbiont.

Among the diverse organisms that participate in chemosynthetic symbioses is *Ifremeria nautilei* (*I. nautilei*), a gastropod found surrounding the deep-sea hydrothermal vents of the Lau, Manus, and North Fiji Basin (Waren and Bouchet, 1991). *Ifremeria* is known to associate with a sulfur-oxidizing(thiotrophic) γ-proteobacterium, but is now suspected to engage in a dual symbiotic partnership with a secondary methane-oxidizing (methanotrophic) symbiont (Borowski et al, 2012), although the abundance and distribution of the diverse symbiont types in *Ifremeria* remains unclear.

The concentration of inorganic chemicals used for chemosynthetic symbioses tend to be heterogeneous over small (centimeters to meters) spatial scales in the vent environment, as well as temporally dynamic. This variability results in the production of gradients in the electron donors available to the chemosynthetic holobiont, the collective "organism" that is comprised of both host and symbionts. For a microbial symbioses to be successful, the holobiont must have a means by which to adapt to these gradients.

Understanding how a symbiosis changes in response to gradients of electron donor availability offers significant insight into the interactions between symbiont partners, as well as the environmental conditions and physiological mechanisms necessary to sustain that partnership. One way to examine these changes is through the measurement of alterations in the community composition and activity of the bacterial fraction of the holobiont. Little is known about how symbiont physiology changes in response to the types of electron donors available in their environment. This is doubly so in symbioses involving multiple, functionally-distinct symbiont types. Our understanding of these associations has been limited due to the difficulty associated with culturing chemosynthetic symbionts outside of their host organism, and manipulating the holobiont under experimental conditions. However, recent advancements in culture-independent methods have provided a means to circumvent this challenge. The advent of high-throughput sequencing (HTS) and meta "omic" analyses in particular have afforded the opportunity to investigate the molecular mechanisms sustaining chemosynthetic symbioses.

Given that *Ifremeria* may be involved in a dual symbiont partnership, it may serve as a unique model for examining how the physiology and composition of endosymbiont communities change across gradients of electron donor availability and type. It may also further the understanding of the environmental/host dynamics under which a dual-symbiont community may be maintained. Here, we use 16S rRNA gene amplicon and metatranscriptomic sequencing to compare differences in symbiont type and activity in the bacterial communities of individual *Ifremeria* exposed to variations in electron donor concentration and type.

Chemosynthetic Symbioses

The relationship between *Ifremeria* and its symbiont is part of a subset of microbial symbioses known as chemosynthetic symbioses. Chemosynthetic symbioses involve chemoautotrophic (obtain energy through the oxidation of electron donors) bacteria and a variety of invertebrate host fauna (Stewart et al, 2005). These symbioses are commonly found in reducing environments, such as cold seeps, whale falls and deep-sea hydrothermal vents. The host organism provisions reduced chemical species, either reduced sulfur or methane, from its surroundings for its symbionts. The symbionts then use those electron donors as a source of energy for carbon fixation. The fixed carbon is delivered to the host for use as a source of nourishment (Dubilier et al, 2008).

Ifremeria nautilei

Ifremeria nautilei, a gastropod of the provannid family, was first discovered surrounding the hydrothermal vents of the Lau, Manus and North Fiji Basin of the Western Pacific in the early 1990s. Like many fauna found surrounding deep-sea hydrothermal vents, *Ifremeria* participates in a chemosynthetic symbioses. Early research suggested that *Ifremeria* is partnered with only one symbiont type, a thiotrophic γ-proteobacteria (Windoffer and Giere, 1997); however, recent studies of the host snail's microbial consortium,based on transmission electron microscopy (TEM) and Fluorescence In-Situ Hybridization (FISH), suggest that it may also be partnered with a novel methanotrophic α-proteobacteria (Borowski et al, 2012). The methanotrophs and thiotrophs are housed intracellularly (indicating that they are endosymbionts) within the gill filaments of *Ifremeria's* whorl, where they may come into contact with methane and reduced sulfur in the surrounding water of the vent habitat (Windoffer and Giere, 1997). The symbionts differ in spatial location, as well as abundance within the gill filaments. The thiotrophs occupy the majority of the gill filament along its central axis in high

abundance, while the methanotrophs are located on the anterior and posterior fringe of the gill in a much lower abundance (Borowski et al, 2012).

Thiotroph-methanotroph "dual" chemosynthetic symbioses have been described for deep-sea mussels of the genus *Bathymodiolus*, and are thought to be an adaptation to support the holobiont under periods of nutrient stress (Distel et al, 1995). The circumstances under which a dual-symbiont partnership are maintained still poorly understood; however populations of *Ifremeria* in certain vent habitats may have adopted multiple symbiont types to exploit a similar strategy.

CHAPTER 2

MATERIALS AND METHODS

Sample Collection and Nucleic Acid Extraction

Sample collection and experimental incubations were performed by individuals of Peter Girguis' lab at Harvard University. Individuals of *I. nautilei* were collected from hydrothermal vent sites within the Lau Basin. Upon retrieval, individuals were held in ambient temperature water for approximately nine hours, and then transferred into pressurized containment vessels composed of an inflow and outflow valve in which sterile seawater could flow. The individual groups were provided with alternate types of reduced sulfur in the following treatments: 300µM hydrogen sulfide (H_2S), 100µM H_2S and 300µM thiosulfate (S_2O_3). One group, the negative control, was not provided with a source of reduced sulfur. C^{13} isotope incorporation was used to estimate rates of carbon fixation among individuals (C-fixation measured by Girguis lab). Following exposure to the treatment conditions, individuals were removed from the containment vessels and dissected, with symbiont-containing gill tissue placed into RNAlater® and stored at -80 ^{0}C until RNA extraction. Total DNA and RNA containing host and symbiont fractions was extracted from symbiont-containing gill tissue of each snail as described in Beinart 2014, using the theAutoGenprep 965/960 Tissue DNA Extraction kit/system (AutoGen, Inc.) and TRIzol (Invitrogen Inc), respectively.

Sample Processing

DNA

Sequencing of amplicons of the bacterial 16S rRNA gene was used to analyze differences in symbiont community composition between individual *Ifremeria* in each treatment. The

hypervariableV4 region of the 16S rRNA gene was amplified from total DNA extracts in duplicate PCR reactions using barcoded, universal primers, as in Kozich et al 2013.Thermal cycling conditions were as follows: Denaturation at 94 ^{0}C (3 min) 35 cycles of amplification at 94 ^{0}C (45 s), primer annealing at 52 ^{0}C (45 s) and primer extension at 72 ^{0}C (90 s). The final primer extension was at 72 ^{0}C (10 min). PCR products were visualized using gel electrophoresis to verify size and purified using Axygen's PCR Cleanup Kit for Amplicons. Following purification, amplicons were pooled at equimolar concentration, quantified, and sequenced on the IlluminaMiSeq platform.

RNA

Metatranscriptomic sequencing was used to determine differences in relative mRNA transcript abundances between treatments.RNA was linearly amplified and converted to cDNA using the ScriptSeq™ v2 RNA-Seq Library Preparation Kit (epicentre). Barcoded, purified cDNA was pooled and sequenced on the IlluminaMiSeq platform.

Sequence Analysis

Following sequencing, barcoded, paired-end datasets for both DNA and cDNA were de-multiplexed, filtered to remove low quality reads and merged using a custom Perl script.

16S rRNAAmplicons

Amplicons were analyzed using the software QIIME (Caporaso et al, 2010). Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, with taxonomy assigned to OTU clusters using the Ribosomal Database Project (RDP) classifier, trained using the Greengenes database, in QIIME.

Metatranscriptome

Preliminary metatranscriptome data were analyzed for a subset of the total number of samples.Reads matching ribosomal RNA genes were identified and separated from protein-coding reads using the program riboPicker (Schmieder et al, 2012). 16S rRNA reads were analyzed using MG-RAST (Meyer et al, 2008) to determine the activity of symbiont types within individual *Ifremeria*. Protein-coding gene sequences were identified among the remaining non-rRNA reads by BLASTX searches against the NCBI non-redundant protein database. BLASTX matches to prokaryotic genes above a bit score of 50 were retained and classified according to functional category, as well as taxonomic identity, using the program MEtaGenomeANalyzer 4 (MEGAN 4; Huson et al, 2012). Genes corresponding to sulfur oxidation (sox) and methane oxidation (formaldehyde assimilation) were chosen over other functional categories for preliminary analysis.

Statistical Analysis

Kruskall-Wallis Analysis of Variance (ANOVA) was used to calculate statistically significant differences between the carbon incorporation rates measured from each individual *Ifremeria***.** A Mann-Whitney U test was used to calculate statistically significant differences between the relative abundance of symbiont types, in each individual *Ifremeria*.

CHAPTER 3

RESULTS

^{13}C Stable Isotope Analysis

The highest and lowest average rates of carbon incorporation were seen in the 300 μM S_2O_3 and 100 μM H_2S treatments, respectively (Table 1). The Kruskal-Wallis ANOVA did not reveal significant differences ($p<0.05$) in carbon incorporation rates between treatments; however, variances were high among treatments and a more robust statistical test will need to be utilized to efficiently analyze these data.

Table 1 Carbon Incorporation Rates

Conditions	Individual	Carbon Incorporation Rate (umol/g/hr)	Mean	Variance
300 µM H_2S	3	0.00	3.74	5.12
	4	1.64		
	5	9.57		
100 µM H_2S	1	0.00	0.306	0.365
	2	0.21		
	3	0.71		
300 µM S_2O_3	1	4.22	6.65	2.25
	2	8.67		
	3	7.06		
Control	1	0.00	0.00	0.00
	2	0.00		
	3	0.00		

16S rRNA Amplicons

A total of 575,737 16S rRNA gene sequences were generated from all treatment conditions following processing for quality control, with highest number of OTU counts per individual at 163454 and the lowest number of OTU counts per individual at 27877 (Table 2)*.As expected, the 16s rRNA amplicon analysis at the level of phylum indicates that the symbiont community of each individual *Ifremeria,* across all treatments, were dominated by γ-proteobacteria (81.2-99.6% of total reads; Figure 1). However, at least two distinct symbiont lineages were observed within the γ-proteobacteria. In all host individuals, the majority (81.2-99.6%) of γ-proteobacteria were most closely related to members of the thiotrophic Chromatiales. In a subset of individuals,0.003-17.5% of all reads clustered within OTUs related to methanotrophic members of the Methylococcales(Figure 2). The results of the Mann-Whitney U indicate that the differences in the relative abundance of each symbiont type (thiotroph versus methanotroph) are significantly different ($p=0.008$). Interestingly, Methylococcales were not detected in any of the individuals in the 300 μMH_2S treatment; however, in all other treatments, two unique populations of Methylococcales were present.

*Individuals in the control group needed to be excluded from these results, as the DNA corresponding to the group that was received was not from Ifremeria' gill extracts.

Table 2 OTU Statistics Summary

Conditions	Individual	OTU Counts/Individual
300 μM H_2S	3	31885
	4	163454
	5	27877
100 μM H_2S	1	101979
	2	59485
	3	51887
300 μM S_2O_3	1	38693
	2	48590
	3	51887
Total		575737

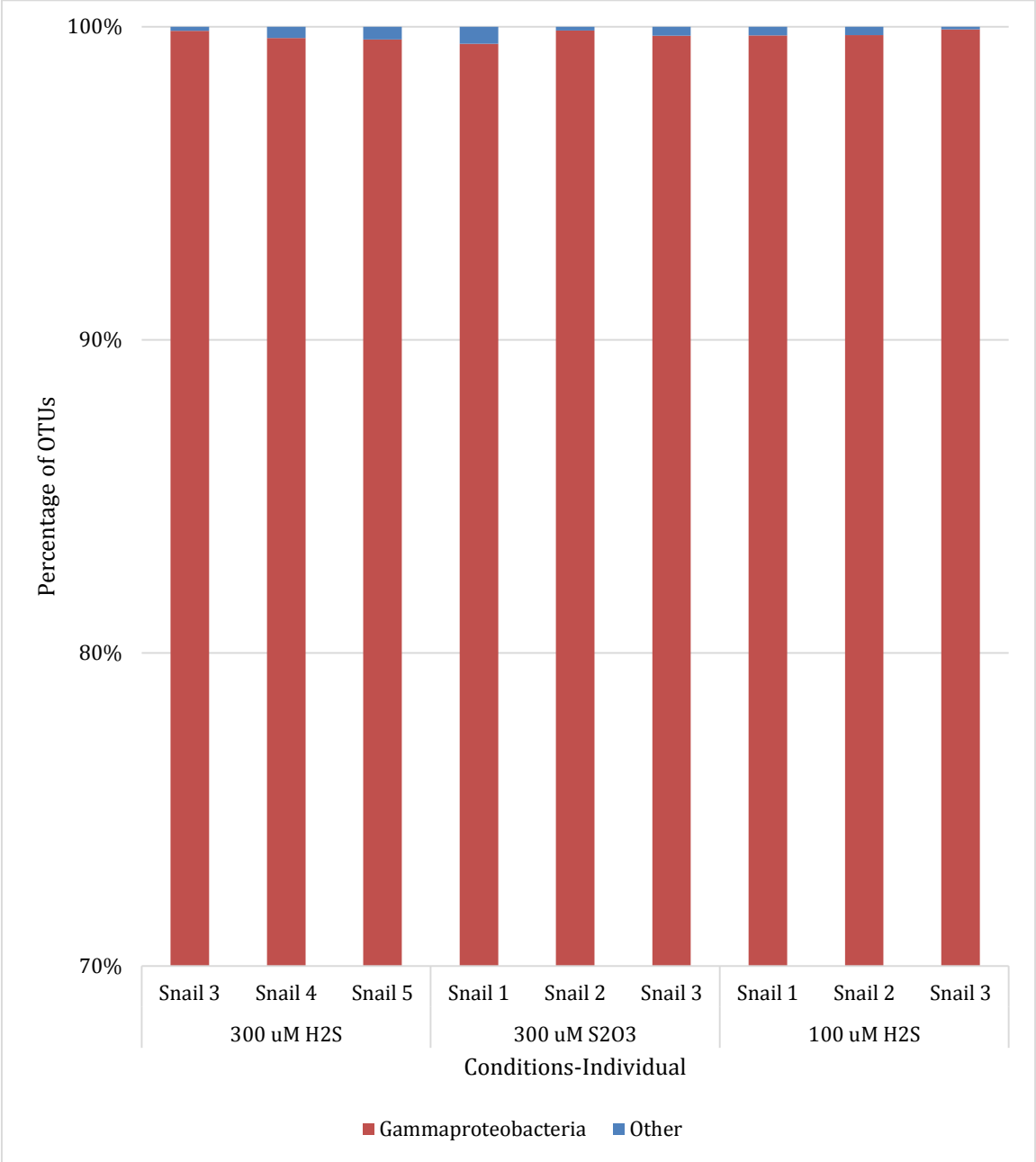

Figure 1 Symbiont Community Diversity-Phyla Relative abundance of OTUs corresponding to bacterial phylum within symbiont communities of individual *Ifremeria* as a percentage of total OTUs/sample.

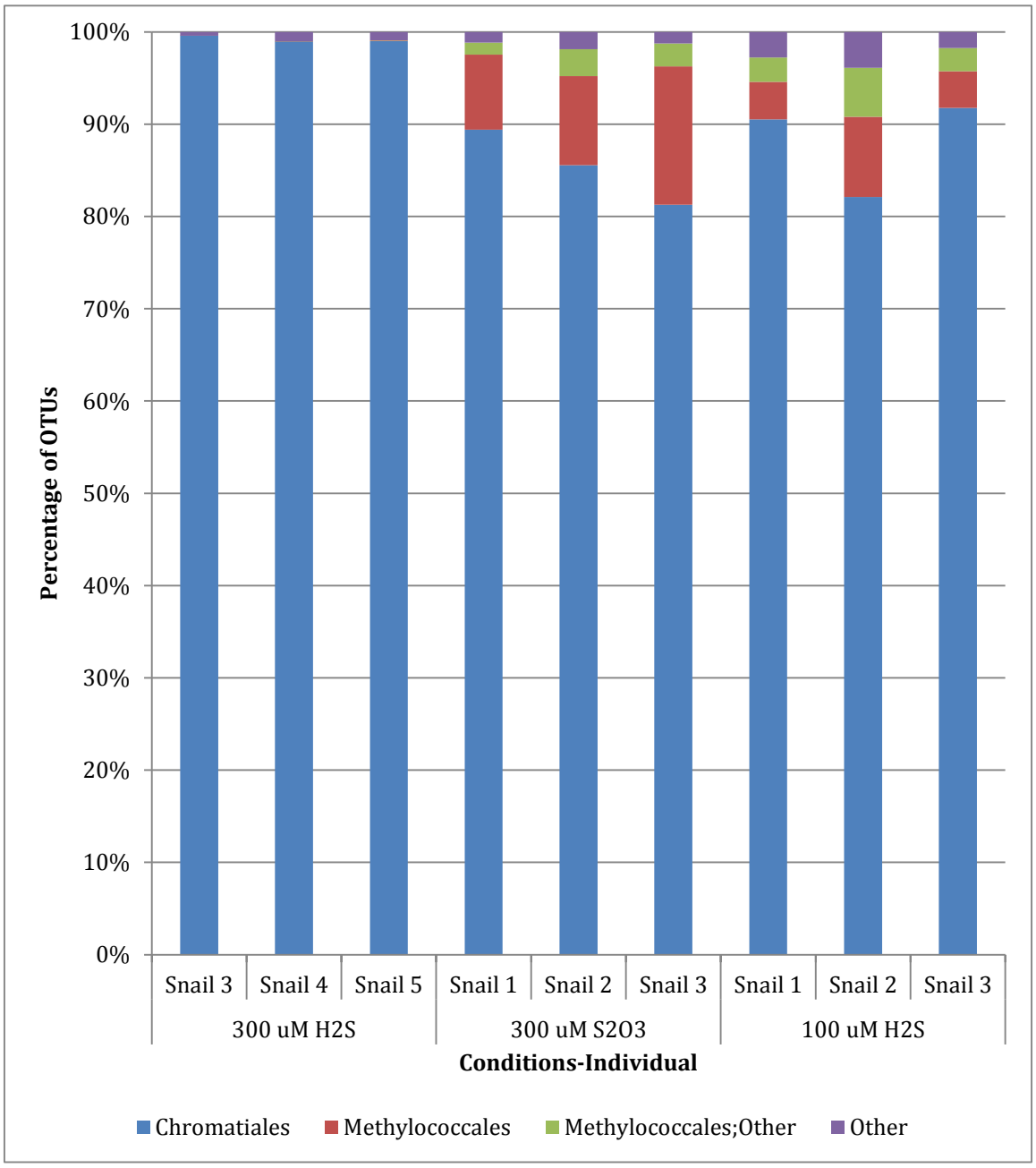

Figure 2 Symbiont Community Diversity-Species Relative abundance of OTUs corresponding to bacterial species within symbiont communities of individual *Ifremeria* as a percentage of total OTUs/sample.

Metatranscriptome

The results of the annotation of the 16S rRNA fraction of the metatranscriptome data mirror those of the 16S rRNAamplicon analysis; individual *Ifremeria*, across all treatments, were associated with two symbiont types: methanotrophic Methylococcales and thiotrophic Chromatiales. Chromatiales represented 99.45-99.97% of all transcripts, while Methylococcales represented 0.01-3.96% of all transcripts (Figure 3). Although the annotation did not distinguish between sub-populations of Methylococcales, Methylococcales were still absent from the high hydrogen sulfide treatment (<0.01% of all transcripts).

Preliminary annotation of the mRNA fraction of the symbiont community of an individual in the 300µM S_2O_3 treatment revealed the expression of genes corresponding to both thiotrophy (sox) and methanotrophy (formaldehyde assimilation; Figure 4). Transcripts matching genes corresponding to thiotrophy were in greater relative abundance (0.79% of all reads), than those corresponding to methanotrophy (0.079% of all reads).

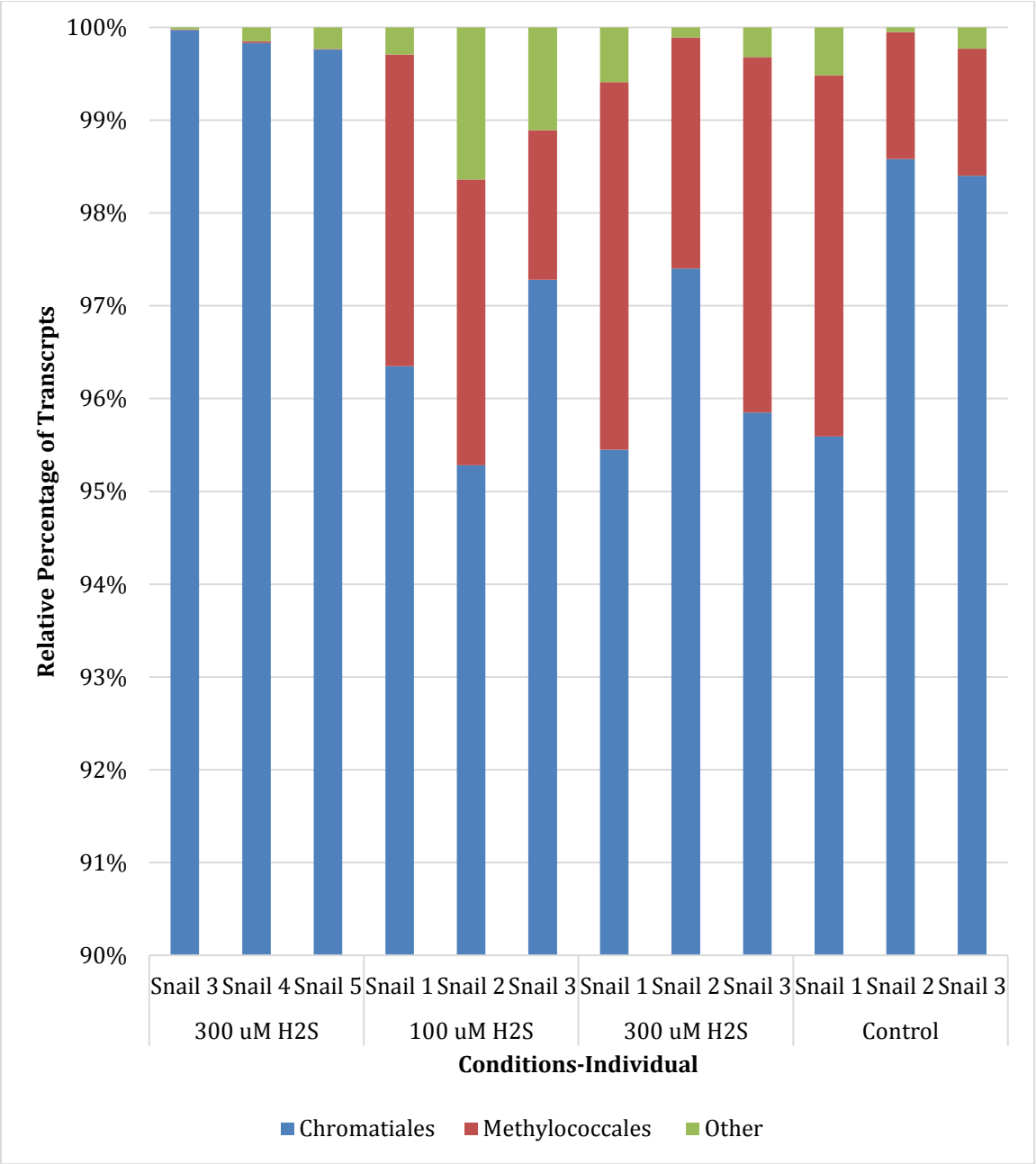

Figure 3 Activity of Symbiont Community Composition Relative abundance of rRNA transcripts corresponding to bacterial species within symbiont communities of individual *Ifremeria* as a percentage of total transcripts/sample.

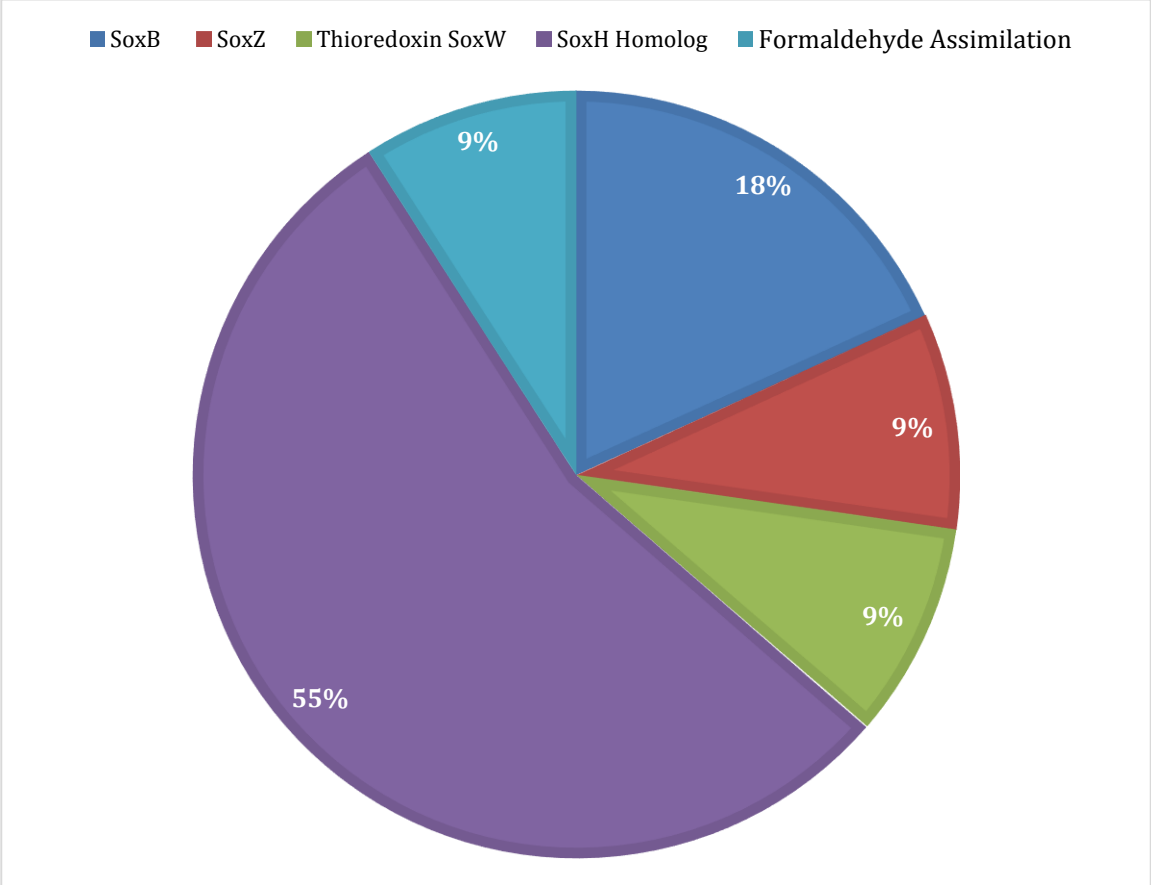

Figure 4 Reads Corresponding to Thiotrophy and Methanotrophy A comparison of the Relative Percentage of reads corresponding to thiotrophy and methanotrophy in an individual in the the 300 µM S_2O_3treatment.

CHAPTER 4
DISCUSSION

Ifremeria's Community Composition and Potential Transmission Strategy

Amplicon analyses revealed two major symbiont lineages within the phylum γ-proteobacteria: putative sulfur-oxidizing symbionts of the Chromatiales and methane-oxidizing symbionts of the Methylococcales. Of these, Chromatiales symbionts dominated, consisting of a single operational taxonomic unit (OTU) representing 81.2-99.6% of the symbiont population. Methylococcales symbionts were represented by two distinct OTUs (0.003-17.5% of sequences) and, excluding those exposed to the 300μM hydrogen sulfide treatment, were present in all host individuals. Similar patterns were seen in the rRNA transcripts removed from the individual's metatranscriptome. These results resolve the ambiguity regarding the composition of Ifremeria's symbiont community and confirm that *Ifremeria* is capable of hosting two symbiont-types*:* thiotrophs and methanotrophs. Interestingly, the methanotrophs described here are of the phylum γ-proteobacteria, while the methanotrophs described previously were considered to be a novel α-proteobacteria. This, coupled with the presence of two populations of methanotrophs seen in the 16S rRNA amplicon analysis, implies that *Ifremeria* may obtain its symbionts from its environment, or horizontally. Vertical transmission, the transmission of endosymbionts from parent to offspring through the germ line, induces symbiont community bottlenecks that result in symbiont community clonality with the end goal of aligning symbiont fitness interestoreduce inter-symbiont competition within the host (Bergstrom and Pritchard 1998). Endosymbiont clonality occurs less often in communities that are horizontally transmitted, where the host may acquire multiple strains of its symbiont type(s) and does not use the germ line to induce symbiont bottlenecks.

The possibility of *Ifremeria* utilizing horizontal transmission may also explain the population-level variability seen hereand in previous characterizations of its symbiont community. The symbiont community composition of individual *Ifremeria* may be dependent on, or "sensitive to" the chemical gradients in the host's surroundings. This "sensitivity" is most evident in the 300 μM H_2S treatment, in which the methanotrophic fraction of the symbiont community was completely absent from each individual's community. This implies one of three possibilities: either the methanotrophic populations were out-competed by their thiotrophic co-symbionts, died of sulfur toxicity, or were ejected from the host, because they were perceived as "cheaters." Each possibility offers insight into the mechanisms and conditions necessary to sustain a dual-endosymbiotic relationship such as *Ifremeria's*. When dual chemosynthetic symbioses were first discovered in *Bathymodiolus*, they seemed to be an evolutionary and ecological anomaly. This is largely due to the potential for co-symbiont competition within the host organism,and the emergence of"cheaters," symbionts that "act" in their own self-interest over the holobiont's. Both are energetically costly for the host (Distel et al 1995); however, hosting two distinct symbiont types also provides a unique strategy for adapting to times of nutrient stress. *Ifremeria* located in vent environments that are prone to transient shifts in chemical availability would benefit from housing two symbionts that utilize different methods of carbon fixation; when one symbiont has no electron donor available to it, the co-symbiont may act as a "backup" to supply the host with carbon. Based on the patterns of symbiont community diversity seen here, periods of nutrient stress are likely to destabilize a dual-symbiont community, opening up the possibility for co-symbiont competition and cheaters.

Metatranscriptomic Changes of *Ifremeria's* Symbiont Community and Future Directions

Of the treatments provided with electron donors, the highest and lowest average rates of carbon incorporation were seen in the 300 µM S_2O_3 and 100 µM H_2S treatments, respectively.Although the changes were not significant ($p=0.08$), significant changes in gene expression between individuals in each treatment may be present. Preliminary results of the metatranscriptome analysis confirm the expression of genes from both symbiont pools, including genes mediating sulfur oxidation and methane oxidation, despite an assumed lack of methane in the treatments. Genes for sulfur oxidation were ten-fold higher in abundance than those for methane oxidation. Regrettably, the small-fraction of metatranscriptomic data presented here is not comprehensive enough to address how the gene expression of *Ifremeria's* endosymbionts changes in response to gradients of electron donor concentration and type. Annotating the remaining mRNA reads and investigating any potential source of the methane-oxidizing signal mentioned previously will be the next steps in completing this analysis.

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